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Biomarker profiling in plants to distinguish between exposure to chlorine gas and bleach using LC-HRMS/MS and chemometrics

Mirjam de Bruin-Hoegée [a,b,*], Marcel J. van der Schans [b], Jan P. Langenberg [b], Arian C. van Asten [a,c]

[a] van 't Hoff Institute for Molecular Sciences, Faculty of Science, University of Amsterdam, P.O. Box 94157, Amsterdam 1090GD, the Netherlands
[b] TNO Defence, Safety and Security, Dep. CBRN Protection, Lange Kleiweg 137, Rijswijk 2288GJ, the Netherlands
[c] CLHC, Amsterdam Center for Forensic Science and Medicine, University of Amsterdam, P.O. Box 94157, Amsterdam 1090GD, the Netherlands

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ABSTRACT

Since its first employment in World War I, chlorine gas has often been used as chemical warfare agent. Unfortunately, after suspected release, it is difficult to prove the use of chlorine as a chemical weapon and unambiguous verification is still challenging. Furthermore, similar evidence can be found for exposure to chlorine gas and other, less harmful chlorinating agents. Therefore, the current study aims to use untargeted high resolution mass spectrometric analysis of chlorinated biomarkers together with machine learning techniques to be able to differentiate between exposure of plants to various chlorinating agents. Green spire (*Euonymus japonicus*), stinging nettle (*Urtica dioica),* and feathergrass (*Stipa tenuifolia*) were exposed to 1000 and 7500 ppm chlorine gas and household bleach, pool bleach, and concentrated sodium hypochlorite. After sample preparation and digestion, the samples were analyzed by liquid chromatography high resolution tandem mass spectrometry (LC-HRMS/MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS). More than 150 chlorinated compounds including plant fatty acids, proteins, and DNA adducts were tentatively identified. Principal component analysis (PCA) and linear discriminant analysis (LDA) showed clear discrimination between chlorine gas and bleach exposure and grouping of the samples according to chlorine concentration and type of bleach. The identity of a set of novel biomarkers was confirmed using commercially available or synthetic reference standards. Chlorodopamine, dichlorodopamine, and trichlorodopamine were identified as specific markers for chlorine gas exposure. Fenclonine (Cl-Phe), 3-chlorotyrosine (Cl-Tyr), 3,5-dichlorotyrosine (di-Cl-Tyr), and 5-chlorocytosine (Cl-Cyt) were more abundantly present in plants after chlorine contact. In contrast, the DNA adduct 2-amino-6-chloropurine (Cl-Ade) was identified in both types of samples at a similar level. None of these chlorinated biomarkers were observed in untreated samples. The DNA adducts Cl-Cyt and Cl-Ade could clearly be identified even three months after the actual exposure. This study demonstrates the feasibility of forensic biomarker profiling in plants to distinguish between exposure to chlorine gas and bleach.

1. Introduction

Chlorine is a toxic dual-use chemical that was first employed as a chemical weapon during World War I [1]. More recently, it has been used multiple times as a weapon in the Syrian Arab Republic. The Fact-Finding Mission of the Organisation for the Prohibition of Chemical Weapons (OPCW) reported that chlorine gas was likely used in 14 instances [2–5], but it is expected that more than 300 chemical attacks with chlorine gas have occurred [6]. Although chlorine is not scheduled in the Chemical Weapons Convention, any use of a chemical to cause intentional death or harm through its toxic properties on life processes is prohibited. Exposure to chlorine inflicts injury mainly in the respiratory tract [7,8]. The acute exposure guideline level associated with mild irritation of mucous membranes (AEGL-1) for a 30-min exposure is 1.5 mg/m^3 [9]. Exposure to a moderate concentration of 9 mg/m^3 for 30 min (AEGL-2) leads to eye and throat irritation, vomiting, inflammation, and the formation of blisters. A severe 30-minute exposure at 81 mg/m^3 (AEGL-3) can result in cell death, pulmonary edema, or a

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* Corresponding author at: van 't Hoff Institute for Molecular Sciences, Faculty of Science, University of Amsterdam, P.O. Box 94157, Amsterdam 1090GD, the Netherlands

E-mail address: mirjam.debruin@tno.nl (M. de Bruin-Hoegée).

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sudden death due to narrowing of the airways [9].

Forensic research into the alleged use of chlorine gas as a chemical weapon is challenging. Several biomarkers have been found, mainly based on *in vivo* and *in vitro* studies, in the lungs [10–12], blood [13–17], nails [18], bronchoalveolar and nasal lavage fluid [19,20], and hair [21, 22]. The protein adducts 3-chlorotyrosine (Cl-Tyr) and 3,5-dichlorotyrosine (di-Cl-Tyr) have also been found in non-human biological samples [23]. In general, this type of evidence is abundant and easier to collect and transport. Other amino acids that might potentially become chlorinated are tryptophan [24], lysine, and valine [25]. In addition, chlorination of the DNA-adducts adenine and cytosine might occur [26,27]. Interestingly, the pH influences the formation of the specific isomer [26].

A disadvantage of the commonly used biomarkers, however, is their formation due to alternative, non-criminal reasons. The chlorinated tyrosine adducts were found in healthy people and at elevated levels in persons with inflammatory diseases [17]. In addition, these biomarkers might be encountered as a result of exposure to other chlorinating agents. Even after exposure to a low concentration of household bleach, chlorinated tyrosine biomarkers were for instance discovered in hair [22]. Also concentrated bleach is a likely candidate to encounter at the scene after a chemical weapons incident, since hypochlorite is often included in general CBRN decontamination procedures [28,29]. Additionally, some rare cases of bleach poisoning were reported, however severe injuries and death only occurred after ingestion of huge amounts [30]. The staging of a chemical attack with household bleach was an alternative scenario proposed by the Syrian Arab Republic and the Russian Federation after a chlorine attack in Douma in 2018 [31]. Although bleach is known as a less reactive chlorinating agent than chlorine gas [32,33], it would be valuable to find novel unambiguous biomarkers that are specific to the actual source of the chlorine, facilitating investigations of alleged use of chemical weapons.

Consequently, the aim of the current study is to explore whether bottom-up proteomics can be used to identify novel post-translational modifications in plants to distinguish between exposure to chlorine and other chlorinating agents. Three model vegetation species were selected, originating from various parts of the world. The low-nutrient feathergrass (*Stipa tenuifolia*) originally grows in South and North America [34]. Green spire (*Euonymus japonicus*) is an evergreen plant, native to Asia [35], while the rapidly growing weed stinging nettle (*Urtica dioica)* is native to Europe [23]. The plants were exposed to three concentrations of chlorine gas and concentrated sodium hypochlorite (NaClO), pool bleach (<15% NaClO), and household bleach (<5% NaClO). After sample preparation, based on previous research [23], the samples were analyzed by liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS). The compounds were tentatively identified with the software package Compound Discoverer and the data was processed by various machine learning methods to reduce data dimensionality and maximize discrimination between the different groups. The identity of a selection of markers was verified by using commercially available or synthetic reference standards and proton nuclear magnetic resonance (^{1}H NMR). The present work demonstrates the potential of using untargeted high resolution mass spectrometric analysis of plant-based samples to discover new biomarkers that can differentiate between exposure to various chlorinating agents.

2. Experimental

2.1. Safety

The experiments, including chlorine gas exposure, were performed in a fume hood by trained personnel. The chemicals were handled in a leak-tight containment and personal protection measures, such as lab coats, gloves, and safety glasses were worn.

2.2. Chemicals and materials

Ammonium bicarbonate (ABC), calcium hypochlorite ($Ca(ClO)_2$), 3-chloro-L-tyrosine, formic acid, 5-chlorocytosine (Cl-Cyt), and protease from Streptomyces griseus (pronase, ≥3.5 units per mg solid) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Acetonitrile (ACN) was purchased from Biosolve (Valkenswaard, The Netherlands). Hydrochloric acid (HCl), 2-amino-6-chloropurine (Cl-Ade), and 4-chloro-D-phenylalanine (Cl-Phe) were obtained from ThermoFisher Scientific (Landsmeer, The Netherlands) while 3,5-di-chloro-L-tyrosine (di-Cl-Tyr) was purchased from BOC Sciences (London, UK). Dopamine was obtained from abcr GmbH (Karlsruhe, Germany). Additionally, MilliQ water (SimPak® 1) was used. The purities of the chemicals exceeded 95%. Feathergrass (*Stipa tenuifolia*) and green spire (*Euonymus japonicus*) were purchased from Intratuin and Nettle (local plant, Rijswijk) originated from the Netherlands. Concentrated sodium hypochlorite (10–20% NaOCl) with 60–185 g/L active chlorine was obtained from Boom (Meppel, The Netherlands). Three commercial household bleaches (< 5% NaOCl) were obtained from local grocery stores Albert Heijn (AH and Glorix) and Dirk (1deBeste). Pool chlorine containing 12.5 – 15% NaOCl was obtained from bol.com (B-care, Smartchim, and Huchem).

2.3. Exposure of the plants

A total of 78 plants were treated with either chlorine gas or bleach and 12 non-exposed plants were evaluated. First, leaves of each plant species were exposed to 7500 ppm chlorine gas (n=3–6, referred to as 'chlorine, high'), which was generated by the reaction of 50 mg $Ca(ClO)_2$ and 1 mL of 12 M HCl, similar to the method applied by de Bruin-Hoegée et al. [17] (Fig. 1A). After reaction, the leaves were left in the closed 2 L glass vial for 30 minutes. It should be noted that this concentration is calculated for a 100% reaction yield. Most likely a lower concentration was generated since some of the $Ca(ClO)_2$ was shielded by chlorine bubbles and remained in the vial after the reaction. In addition, each plant species was exposed to 1000 ppm chlorine gas (n = 6) generated from a cylinder for a long exposure duration of 1 hour at 1 L/min (referred to as 'chlorine, long') or a short exposure duration of 20 minutes at 0.5 L/min (referred to as 'chlorine, short') as depicted in Fig. 1B.

The plants were also exposed to three commercial household bleaches (< 5% NaOCl), three brands of pool chlorine (12.5–15% NaOCl), and concentrated NaOCl (10–20%, n=3–6). This was conducted by fully immersing a part of the living plant in the liquid for 30 minutes (Fig. 1C). Afterwards the leaf was dried, and the pH of the slightly moist plants was measured before and after exposure.

2.4. Protein precipitation and digestion

After exposure, the sample preparation and pronase digestion were applied according to the method described by de Bruin-Hoegée et al. [23]. The only exception was the exclusion of the washing step, to prevent the loss of potential biomarkers. A total of four living plants exposed to chlorine gas ('chlorine high') were also sampled after three months. The samples were digested and stored at −20 °C for two years before analysis was conducted.

2.5. Chemical analysis

2.5.1. Data-dependent acquisition by LC-HRMS/MS

The samples were analyzed with a Thermo Ultimate 3000 UHPLC equipped with a Waters Acquity HSS T3 C18 column (1.8 µm, 2.1 × 100 mm). The column temperature was maintained at 30 °C with a flow rate of 100 µL/min. Eluent A consisted of 0.2 v% formic acid in MilliQ water. Eluent B was composed of 0.2 v% formic acid in acetonitrile. Gradient elution started at 100% eluent A, ramping to 80% eluent B in

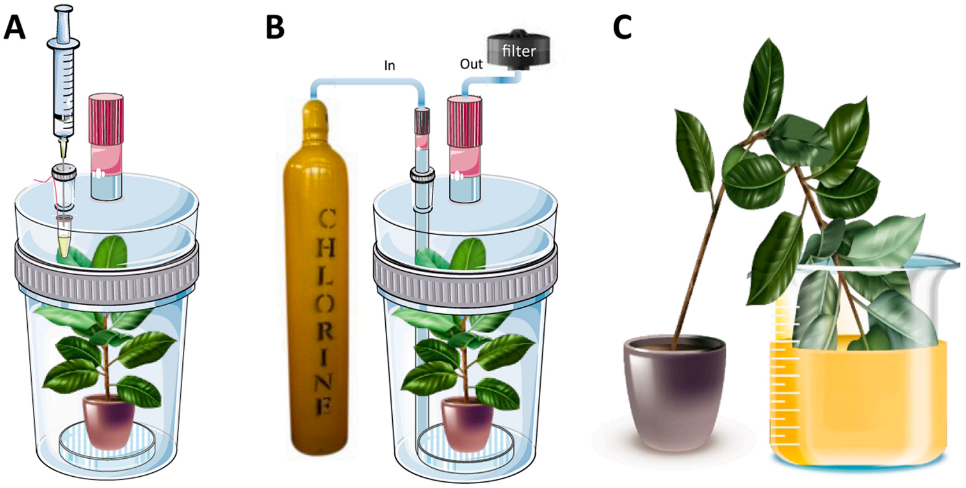

Fig. 1. Schematic experimental set-up for controlled exposure of plants to A) A maximum of 7500 ppm chlorine gas formed by the reaction of 1 mL HCl injected into a vial with 50 mg $Ca(ClO)_2$, B) 1000 ppm chlorine gas generated from a gas cylinder, and C) bleach.

10 min and holding for 5 min. Then equilibrating at 100% eluent A for 1 min. The injection volume was 10 μL. The UHPLC was coupled to a Thermo Scientific Q Exactive Plus Orbitrap MS, which was set to a mass range of *m/z* 50–750 and operated in positive ESI mode. The capillary voltage was set to 3.5 kV, and the source temperature was maintained at 320 °C, the relative sheath gas (nitrogen) flow was 35. Data were first acquired with full scan MS mode. Based on the results obtained, an inclusion list was established using targeted MS/MS in parallel reaction monitoring (PRM) mode. The collision energy was 25 eV for all compounds.

2.5.2. *Targeted analysis by LC-MS/MS*

A selection of the samples was also analyzed with a Waters (Milford, MA, USA) Acquity ultra-high-pressure liquid chromatographic (UPLC) system equipped with a Waters Acquity HSS T3 C18 column (100 ×2.1 mm I.D., 1.8 μm). A volume of 5 μL was injected at 8 °C, after which the analysis was performed at room temperature with a gradient flow of 100 μL/min. Eluent A consisted of 0.2 v% formic acid in MilliQ water. The composition of eluent B was 0.2 v% formic acid in acetonitrile. Gradient elution started at 100% eluent A, holding for 2 min, then ramped to 80% eluent B in 8 min and held for 2 min. Finally, the system was equilibrated at 100% eluent A for 3 min in preparation for the next analysis. The UPLC system was coupled to a Waters Xevo TQ-S triple-quadrupole mass spectrometer, equipped with electrospray ionization operating in positive ionization mode. A capillary voltage of 3.5 kV was applied. The nitrogen cone gas flow was 150 L/h, and the argon collision gas flow was set to 0.19 mL/min. Data was acquired in selected reaction monitoring (SRM) mode and all compounds were analyzed with a single chromatographic method. Table S1 in the Supporting Information shows the chromatographic and mass spectrometric parameters for the biomarkers Cl-Tyr, di-Cl-Tyr, Cl-Phe, Cl-Cyt, chlorodopamine (Cl-dopamine), dichlorodopamine (di-Cl-dopamine), and trichlorodopamine (tri-Cl-dopamine). The identity of these compounds was verified with a synthetic reference standard. The identification was confirmed by comparing retention times, precursor ion, and characteristic fragment ion *m/z* values. Cone voltages of 10–30 V and optimized collision energies of 15–30 eV were used.

2.6. *Data analysis*

Data were first processed with Compound Discoverer 3.3 (Thermo Scientific). Peak areas were calculated by automatic integration of the extracted ion chromatogram of the identified compounds after subtraction of a negative control baseline signal as obtained for non-exposed plants. An inclusion table was established based on the first full-scan results. A large fraction of chlorinated chemicals did not match with a compound in the Chemspider database and only a formula was given. In contrast, for most non-chlorinated chemicals a match was found with the database. Consequently, the non-chlorinated chemicals in the blank were compared to the formulas of unidentified chlorinated chemicals in the treated samples. It was hypothesized that the -OH or -H would be replaced by a chlorine atom. For instance, dopamine ($C_8H_{11}NO_2$) was identified in the blank samples and a compound with formula $C_8H_{10}ClNO_2$ was found in the treated samples. This sample was preliminary identified as dopamine + Cl* and the mass was added to the inclusion table. Only chlorinated chemicals were included in the final selection. Ultimately, the markers are also visible in future research where samples might be exposed to even lower concentrations, therefore small peaks (area < 1E7, <1% of maximum peak) were excluded. In addition, the chemical should have been identified in at least 3 repetitions of either the bleach or chlorine samples. By applying those criteria, the number of compounds was reduced from 13000 to 150. Finally, only compounds were considered that were either tentatively identified by MS/MS or present as non-chlorinated compound in the blank, resulting in 93 markers.

Python 3.9.12 with scikit-learn 1.0.2 and lir 0.1.27 was used for the machine learning analysis. The code written for this research is published under a GNU General Public License [36]. PCA was applied to reduce the dimensionality of the data and identify discriminating markers. QuantileTransformer (quantiles=6) was used for normalizing the data. This method applies a non-linear transformation where the probability density function of each feature follows a uniform distribution and concurrently preserves the rank of the values along each feature. It reduces the impact of outliers, and it also provides results with similar standard deviations within classes for a given feature. The robustness was evaluated by leave-one-group-out validation. Subsequently, two LDA binary models were constructed from the data to distinguish between unexposed and exposed samples and to discriminate between samples treated with chlorine or bleach. In addition, an LDA score plot was used to classify the specific source of exposure. For all models, 3-fold cross-validation using Kernel density estimation (KDE) was applied. Two thirds of the data (containing two types of plant species) was used to train the model and one third (containing one group of plant species) was used to test the performance. After KDE construction, LR values were calculated for the classification of the exposure condition, in a similar way as described by de Bruin-Hoegée et al. [37]. Because limited samples were evaluated, the issue of extrapolation was addressed by artificially reducing the empirical upper and lower bound

(ELUB) for LR systems by a method based on the normalized Bayes error-rate as proposed by Vergeer et al. [38].

3. Results & discussion

3.1. Visual examination of vegetation

After the exposure, the pH of the plants was assessed by bringing it into contact with pH-indicator paper (Merck, pH 1–10). The pH of the blank, chorine exposed, and bleach immersed leaf was approximately 6–8, 2, and 9, respectively. Because of this variation, it is likely that different chemical reactions will occur after chlorine and bleach exposure [26], which can result in different biomarker profiles. Fig. 2 shows the color change of the leaves over time. The leaf that was exposed to chlorine gas turned from green to brown to yellow. Small white spots were visible in the samples immersed in concentrated bleach and after one day the sodium hypochlorite crystallized on the surface of the leaf. It should be noted however, that household and pool bleach resulted in similar discoloration as the leaves exposed to chlorine, which is likely due to other additives in the cleaning solution. Therefore, color change is not an unambiguous indicator of chlorine versus bleach exposure and cannot be used exclusively as a differentiating feature.

3.2. Classification of chlorinating agent

After data optimization, 93 chlorinated compounds were tentatively identified based on the exact mass, MS/MS spectra, and comparison with spectral libraries. An overview is presented in Table S2 in the Supporting Information. The compounds consist of chlorinated adducts of plant fatty acids, protein, and DNA adducts. All except five compounds were found in all species of treated investigated vegetation. In contrast, these specific markers were not found in all species of untreated plants. Most compounds were detected in both chlorinated and bleach exposed samples.

Subsequently, an unsupervised PCA model was constructed using the normalized peak area. Fig. 3 shows the score plot of the two first principal components with grouping of the samples according to their exposure to either chlorine gas or bleach. There is full discrimination between samples treated with chlorine and bleach. However no clear trend with chlorine exposure level or exposure time is visible. This is not necessarily a disadvantage, because it means that similar markers were found for various chlorine concentrations and exposure times, making it better applicable to real-life situations where such variation is expected. The PCA model was found to be quite robust for the given data set, since leaving out one type of exposure did not substantially reduce the explained variance (as indicated in Figure S1 in the Supporting Information).

Additionally, the supervised machine learning method LDA was used for classification. First, a binary LDA model was computed to maximize discrimination between exposed (i.e., treated with bleach and chlorine) and unexposed samples. Figure S2 of the Supporting information shows clear discrimination between the unexposed and bleach or chlorine exposed plants. After indication of exposure, a second binary model was applied to distinguish between chlorine and bleach exposure. Fig. 4 shows the distributions of the LDA test values. Based on the constructed Kernel density estimation, LRs were calculated. The following hypothesis pair was considered: H_1: The plants were exposed to chlorine gas, and H_2: The plants were exposed to bleach. The minimum and maximum LR values were assimilated to the size of the dataset, resulting in ELUB boundaries of $6.8*10^{-2}$ to 12 (Figure S3 of the Supporting Information). Due to the limited amount of data, the LR values were severely underestimated. False positive error rates were 0.7% and false negative error rates were 1.5%, as visualized in Tippett plots in Figure S4 in the Supporting Information. In addition to the binary model, LDA with 3-fold

Fig. 2. Blank nettle leaves (top row), leaves immersed in bleach (2nd row) and leaves exposed to 7500 ppm chlorine gas (3rd row). From left to right: after 0 min, 2 min, 5 min, 10 min, 30 min, 60 min, and 24 hours.

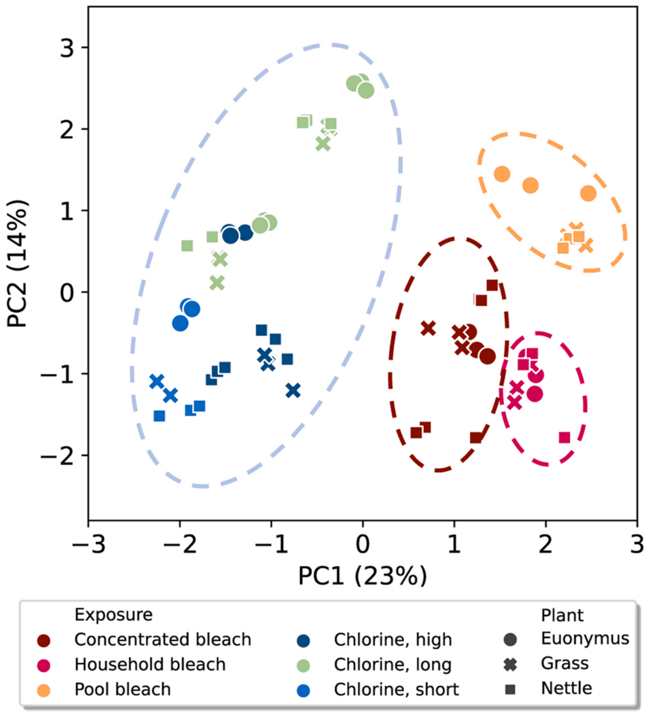

Fig. 3. PCA-score plot based on 93 biomarkers detected by LC-HRMS/MS after chlorine gas or bleach exposure (n=9–18).

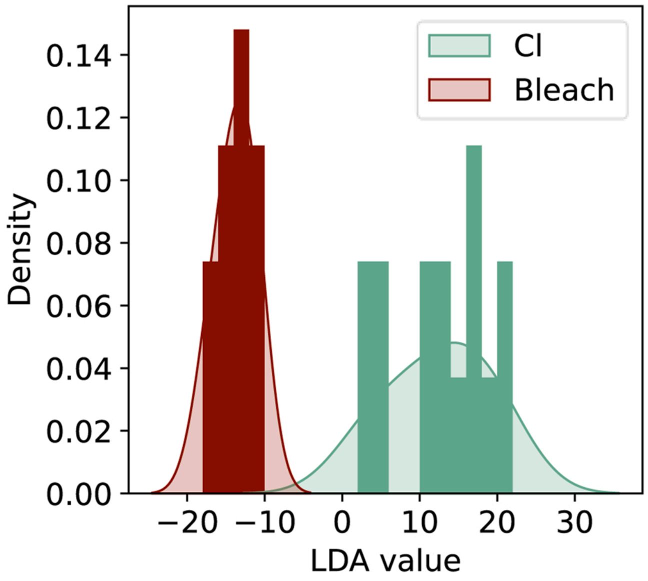

Fig. 4. Distribution of LDA test scores after validation for plants exposed to chlorine gas (Cl) and bleach. The bars represent the frequency of the individual measurements for a given LDA value adding up to 1. The shaded curve is the kernel density estimation with a bandwidth of 0.8 (chlorine) and 1 (bleach).

cross validation was performed to establish the specific source of exposure. Fig. 5 shows the LDA-score plot of the test data with clear discrimination between the various groups. However, it should be noted that for the other two test sets, full separation of the classes was not achieved (Figure S5 of the Supporting Information).

Based on the PCA loadings, compounds can be discovered that contribute significantly to the separation of the classes (Figure S6 in the Supporting Information). In this study, discrimination was not dominated by one specific component. Rather a group of chemicals were found to be relevant markers. The chlorinated amino acid adducts dichlorinated valine, dichlorinated tyrosine, and chlorinated tryptophan are of importance. In addition, the chlorinated form of the natural product benzoic acid constitutes an important feature. Other interesting compounds that were detected are Cl-dopamine, di-Cl-dopamine, and tri-Cl-dopamine, which were only present in the chlorine-exposed samples and not in the bleach-treated samples. In addition, 4-chlorophenol (MCP) and 2,4-dichlorophenol (DCP) are relevant. Interestingly these compounds were also found in concrete samples during the investigation of alleged use of chemical weapons in Douma [31]. It should be noted that no extensively chlorinated phenols, such as trichlorophenol (TCP) and tetrachlorophenol (TeCP), were found. However, the accessibility and reactivity in biological samples might be different compared to concrete and wooden objects, resulting in different degrees of chlorination. In the current research MCP was only detected in the chlorine-exposed samples while the amount of DCP (as indicated by the peak area) was found to be in a 100-fold excess in the chlorine-exposed samples. A selection of markers was further investigated as will be described in more detail in Section 3.3.

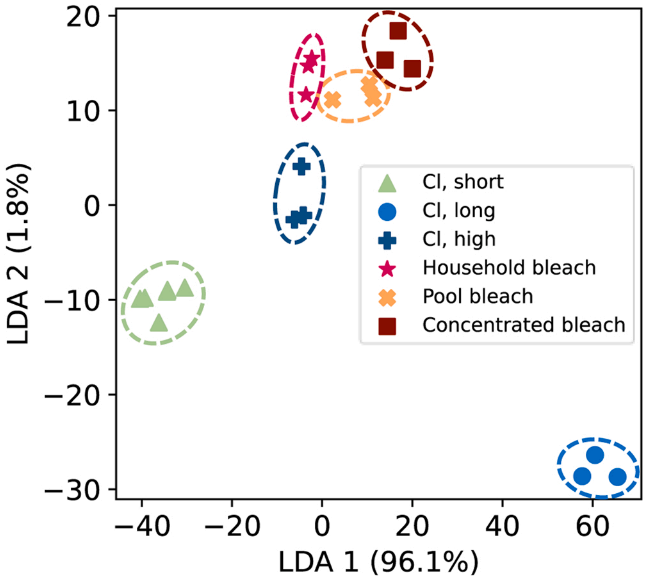

Fig. 5. LDA-score plot after validation for classification of plants exposed to a short duration, long duration, or high concentration chlorine gas and household, pool, or concentrated bleach. Corresponding LDA scores for the first and second discriminant function are shown.

3.3. Identification of novel biomarkers

3.3.1. Amino acid adducts

The tentative identification of selected novel biomarkers was verified by comparison with reference standards. Targeted LC-MS/MS analysis was performed of the exposed plants, commercially available reference standards for Cl-Tyr, di-Cl-Tyr, Cl-Phe, Cl-Cyt, and Cl-Ade, and synthetic reference standards for Cl-dopamine, di-Cl-dopamine, and tri-Cl-dopamine. None of these chlorinated biomarkers were observed in untreated samples. The amino acid adducts Cl-Phe, Cl-Tyr, and di-Cl-Tyr were present in all exposed samples, but a higher concentration was detected in the plants exposed to chlorine gas. Fig. 6 shows the extracted ion chromatograms of the commercially available reference standard for Cl-Phe, nettle exposed to concentrated bleach, and nettle exposed to a high concentration of chlorine gas. The additional peak at a retention time of 6.4 min might be an isomer, such as ortho- or meta-chlorophenylalanine [39]. The mass spectrum of the commercially available reference standard with at least two characteristic mass transitions matched the spectrum of the compound found in exposed plants.

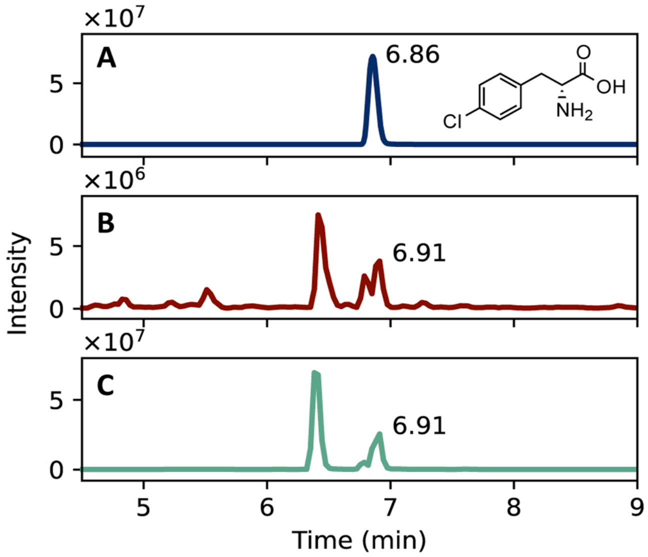

Fig. 6. Extracted ion chromatograms (*m/z* 200.0 → 153.9) of 4-chloro-D-phenylalanine (Cl-Phe) at t_R=6.86 min. A) Commercially available reference standard, B) Nettle exposed to concentrated bleach, C) Nettle exposed to a high concentration chlorine.

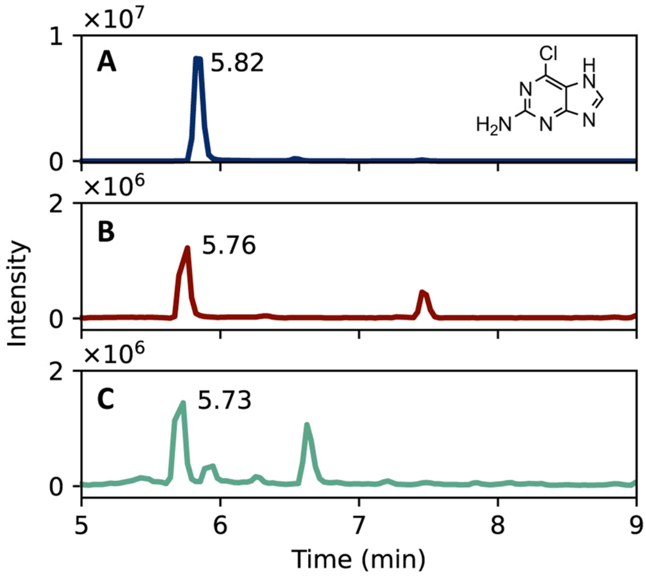

Fig. 7. Extracted ion chromatograms (*m/z* 170.0 → 107.0) of 2-amino-6-chloropurine (Cl-Ade) at t_R=5.82 min. A) Commercially available reference standard, B) Nettle exposed to concentrated bleach, C) Nettle exposed to a high concentration chlorine.

Additionally, the variation in retention time was within 0.2 min, as recommended in the work instruction for the reporting of the results of the OPCW biomedical proficiency tests [40]. An average area ratio of 3:1 was found for chlorine gas exposure compared to bleach exposure. For Cl-Tyr and di-Cl-Tyr which were also identified in previous research [23], the response was respectively 8 and 19 times higher for chlorine exposure (Figure S7 in the Supporting Information). Previous research demonstrated that Cl-Tyr and di-Cl-Tyr could still be detected in living plants and dried leaves three months after exposure [23]. This is invaluable in real-life scenarios where sampling can be delayed. In the current study, the marker Cl-Phe could not be detected anymore after three months. However, it is important to note that the samples were stored for a long period of time, so future research should be conducted to gain a better understanding of the stability of this marker. In the following sections, a ratio of markers will be introduced as a more robust statistic to compensate for varying exposure conditions.

3.3.2. *DNA-adducts*

In addition, two chlorinated DNA-adducts were identified. Fig. 7 shows the extracted ion chromatograms of the DNA-adduct 2-amino-6-chloropurine (Cl-Ade). This biomarker was present in similar abundance in the bleach and chlorine exposed samples. The retention time was slightly shifted, although it is still within the allowed maximum variation. Interestingly, a compound at t_r=7.43 min was 300-fold more abundant in the bleach samples. This could be an isomer of 2-amino-6-chloropurine which is mainly formed after exposure to chlorinating agents with low pH. Also, an isomer with the same mass was preliminary identified at another t_r = 6.62 min, which was more abundant in the chlorine samples. Future research could focus on isolation of unknown markers and structural identification of these markers by NMR. Another DNA-adduct 5-chlorocytosine (Cl-Cyt) was identified (Fig. S8 of the Supporting Information). Contrary to the former DNA-adduct, this compound was detected in a 10:1 ratio in chlorine samples compared to bleach-exposed plants. According to Xiang et al., chlorine substitution on the heterocyclic ring occurs at low pH, while chlorine substitution on the aliphatic amine is more likely at a higher pH [26]. Since they only report on chlorinated oxidation products, the results cannot directly be applied to the non-oxidized chemicals in the current study. Nonetheless, the authors demonstrated that the pH could have a significant effect on the formation of a specific isomer. Therefore, the lower pH could be a possible explanation for the fact that the other isomers were only detected in the bleach samples. Remarkably, these two DNA-adducts were still detected in living plants three months after the actual exposure to chlorine gas. The chromatograms analyzed three months after exposure are presented in Section S4 of the Supporting Information.

In forensic cases, the exposure concentration is often unknown, which makes it challenging to interpret the results. The utilization of one compound as internal standard could function as a normalizer and correct for varying sample conditions and analytical methods. A relatively sensitive parameter would be the ratio of Cl-Cyt/Cl-Ade which is 10 ± 8 (n=5, ±95% confidence interval) times higher for chlorine exposed samples compared to plants treated with bleach. Alternatively, the ratio of di-Cl-Tyr/Cl-Cyt is 3.8 ± 2.8 (n=5, ±95% confidence interval) fold higher for samples treated with bleach compared to plants exposed to chlorine gas. Together these results are promising for a robust and simple assessment of the source of Cl exposure.

3.3.3. *Chlorinated dopamine*

In contrast to the previously described compounds, chlorinated dopamine was only found in chlorine-exposed plants and is consequently a very promising biomarker for chlorine gas exposure. Dopamine promotes the growth of plants under stressful environmental conditions [41]. During drought, salt stress, and nutrient deficiencies, the endogenous dopamine concentration increases [41]. Its content varies amongst different species and its presence is most abundant in fully developed green leaves [42].

Since no commercially available standards exist, the plant samples were compared to dopamine (purity 95%) exposed to chlorine gas. The chlorinated products were separated and purified by LC-MS. Afterwards, the identification was confirmed with NMR and high-resolution mass spectrometric analysis of the exact mass, chlorine isotope patterns, and ion fragmentation. The NMR spectra are shown in Section S5 of the Supporting Information. Fig. 8 displays a distinct chlorination pattern for Cl-dopamine, di-Cl-dopamine, and tri-Cl-dopamine in nettle plants and a single peak was visible for non-chlorinated dopamine as expected. The isotope pattern corresponds to the spectra of the synthetic reference standard for Cl-dopamine. Also, excellent correlation was found for the measured versus the theoretical monoisotopic masses and isotope ratios, with a maximum deviation of *m/z* 0.0011 (Section S6 of the Supporting

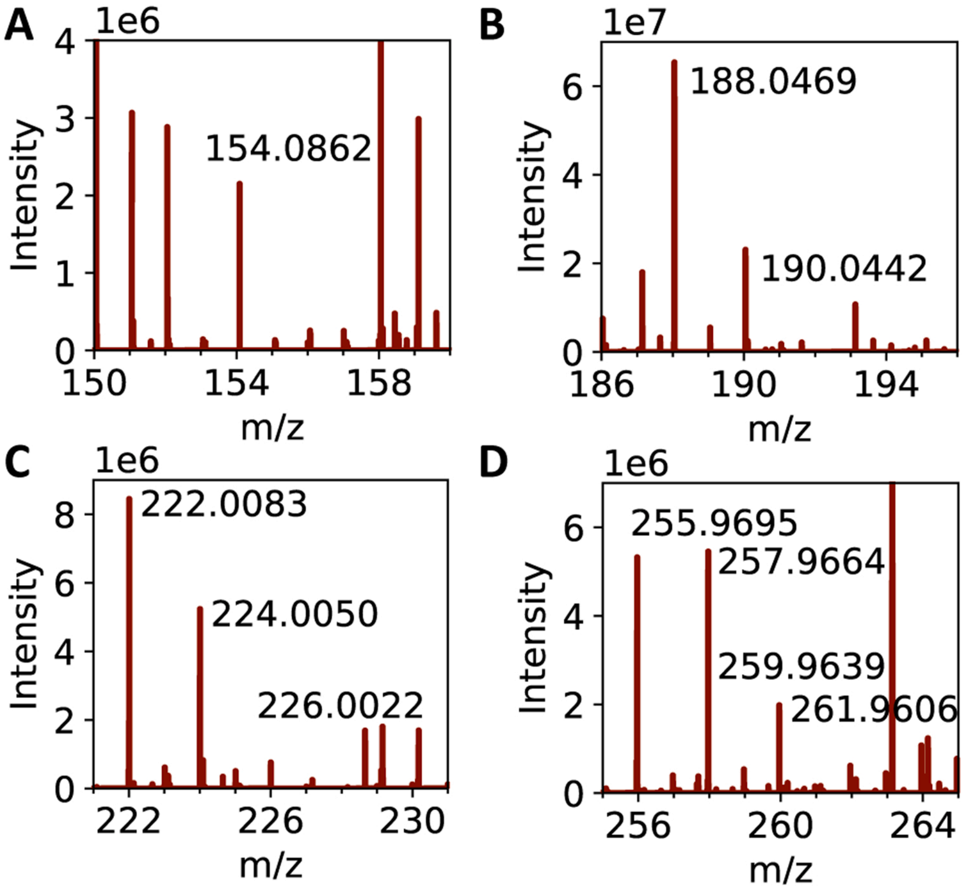

Fig. 8. Full scan MS spectrum (LC-HRMS/MS) of singly charged chlorinated dopamine in nettle plants showing the chlorine isotope pattern. A) Dopamine, B) Cl-dopamine with an isotope ratio of 3:1, C) Di-Cl-dopamine with an isotope ratio of 9:6:1, and D) Tri-Cl-dopamine with an isotope ratio of 9:9:3:1. The pattern and masses correspond to the results observed for the synthetic reference standard presented in Fig. S20, Table S3, and Table S4 of the Supporting Information.

Information).

Fig. 9 shows the extracted ion chromatograms of di-Cl-dopamine.

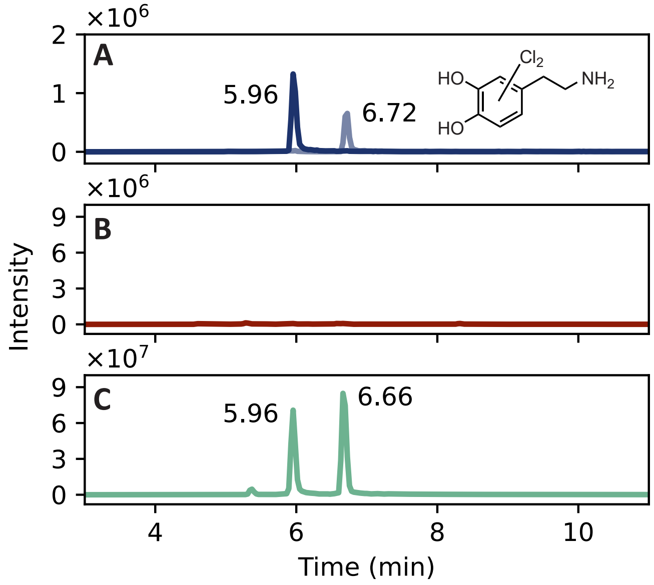

Fig. 9. Extracted ion chromatograms (m/z 222.0 → 205.0) of dichlorodopamine (di-Cl-dopamine) at t_R=5.99 and 6.67 min. A) Synthetic reference standard, B) Nettle exposed to concentrated bleach, C) Nettle exposed to a high concentration chlorine.

The chromatograms for Cl-dopamine and tri-Cl-dopamine are presented in Figs. S9 and S10 in the Supporting Information. The retention time and mass spectrum of the compounds detected in nettle matches with the data of the synthetic reference standards. Tri-Cl-dopamine shows only one peak in the extracted ion chromatogram, while multiple peaks are present for both Cl-dopamine and di-Cl-dopamine. For both peaks comparable mass fragments are observed, which suggests the formation of two structurally similar isomers and not another compound with the same mass. This may be consistent with dopamine that can be chlorinated at different reaction sites in the aromatic part of the chemical. Even three months after exposure di-Cl-dopamine and tri-Cl-dopamine were still identified in the plant samples exposed to chlorine gas and measured by LC-MS/MS (Section S4 of the Supporting Information). Only Cl-dopamine could not be detected anymore. However, these findings are limited since these markers could not unambiguously be identified in all analyzed samples because only one mass transition was found in some samples. Future research is required to gain a better understanding of the stability of chlorinated dopamine markers.

Based solely on the mass spectra no unambiguous identification of chlorinated dopamine could be accomplished (Section S7 of the Supporting Information). It was only revealed that chlorine did not react with the amine group to form stable products, since for all variants the -NH3 fragment was observed. Therefore, ^{1}H NMR analysis was performed to elucidate the chemical structure. The NMR spectra indicate that chlorination occurs in the aromatic ring. By comparison with reference spectra, Cl-dopamine is most likely identified as 4-(2-aminoethyl)-5-chloro-1,2-benzenediol [43,44]. In the present study, the NMR spectra from the isolated compounds corresponding to the different peaks could not be distinguished from each other, so additional research is recommended to determine the exact chemical structure. Since no reference spectra are available for di-Cl-dopamine and tri-Cl-dopamine, the results were compared with predicted spectra. The two peaks of di-Cl-dopamine were preliminary identified as 4-(2-aminoethyl)-3,6-dichlorobenzene-1,2-diol (t_r: 5.96 min) and 4-(2-aminoethyl)-3,5-dichlorobenzene-1,2-diol (t_r: 6.66 min). Presumably the small peak at t_r 5.4 min corresponds to the more sterically hindered 5-(2-aminoethyl)-3,4-dichlorobenzene-1,2-diol. The relatively low amount of synthesized marker limits the possibility to measure the sample with ^{13}C NMR and obtain a more detailed ^{1}H NMR spectrum. Therefore, this study lays the groundwork for future research into the identification of a larger batch of chlorinated dopamines with a broader range of techniques.

4. Conclusions

In this study novel chlorine biomarkers were identified in vegetation using high resolution mass spectrometry with machine learning methods. The methodology proposed can be a valuable tool for the exploration of new biomarkers after contact with exogenous compounds and forms the basis for distinguishing between chlorine and hypochlorite exposure. Principal component analysis and linear discriminant analysis of tentatively identified compounds, such as chlorinated adducts of plant fatty acids, proteins, and DNA adducts, enabled discrimination between chlorine gas exposure and various types of bleach. Distinctive markers that were discovered include dichlorinated valine, chlorinated tryptophan, chlorobenzoic acid, Cl-dopamine, di-Cl-dopamine, tri-Cl-dopamine, 4-chlorophenol, and 2,4-dichlorophenol. Since the chemometric analysis might be harder to implement in forensic laboratory practice, some biomarkers were also verified using commercially available or synthetic reference standards. Ultimately these biomarkers result in unambiguous identification. Unfortunately, most of the verified compounds were detected in both bleach and chlorine samples. Therefore, it remains important to compare case samples with control samples of locations where no threat agent was released. The amino acid adducts Cl-Phe, Cl-Tyr, and di-Cl-Tyr and the DNA-adduct Cl-Cyt were 3–19-fold more abundant in plants exposed to chlorine gas, compared to bleach exposed vegetation. Contrary to this, Cl-Ade was present at similar concentrations in both types of samples, and a preliminary identified isomer of Cl-Ade was even more than 300-fold more abundant in plants exposed to bleach. Consequently, the ratio of Cl-Cyt/ Cl-Ade is a promising parameter where a low value was found for bleach exposure, and a high number indicates chlorine exposed samples. By utilizing a compound ratio, absolute amounts are normalized making this parameter robust to actual exposure conditions. Interestingly, in addition, Cl-dopamine, di-Cl-dopamine, and tri-Cl-dopamine were solely detected in plants exposed to chlorine gas. To our knowledge, these biomarkers have not been reported as markers for chlorine gas exposure before. Lastly, this study demonstrated that biomarkers formed after chlorine exposure in plants are very persistent and can be identified up to three months after exposure. Future research could focus on confirming the identity of more biomarkers with reference standards, to provide extra tools for investigations of alleged use. In addition, lower exposure concentrations could be investigated with a combination of various analytical techniques. Also, the selection of the leaves could be improved by screening with portable detection techniques. In conclusion, promising biomarkers were identified by untargeted high resolution mass spectrometry facilitating incident reconstruction and differentiating between chlorine and bleach exposure.

CRediT authorship contribution statement

Mirjam de Bruin-Hoegee: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Marcel J. van der Schans:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Jan P. Langenberg:** Writing – review & editing, Funding acquisition, Conceptualization. **Arian C. van Asten:** Writing – review & editing, Supervision, Methodology, Conceptualization..

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2024.112022.

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